BARD Final Report IS-4841-15

Novel strategies and methodologies to control and study *Candidatus* Liberibacter diseases

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One year feasibility project

ABSTRACT

Our proposal entitled “Novel strategies and methodologies to control and study *Ca.* Liberibacter diseases” was submitted as a one-year feasibility research grant. The long term goal of our proposal is to develop novel tools for the study of the thus far uncultivable plant pathogen ‘*Ca*. Liberibacter spp.’ and to develop ‘*Ca*. Liberibacter’-resistant plants using induced activation of the plant’s hypersensitive response (HR). The specific objectives for the one-year grant period were to:

I. Identify and validate ‘*Ca*. L.’-specific induced host genes that would serve as candidate promoters for the induced HR approach.
II. Establish and optimize a microfluidic-based system for *in vitro* cultivation and study of Las.

Diseases caused by *Ca*. L. spp. have emerged in recent years as significant threats to multiple agricultural industries. First and far most is the citrus industry in Southeast United States were the bacterium ‘*Ca*. L. asiaticus’ (Las) has seriously damaged all the orange growing groves. Despite its appearance in Florida over a decade ago, no efficient control means were yet successful. Additionally, the inability to culture Las is setting back more significant research on this important pathogen.

During this grant period we conducted experiments to study tomato and potato transcriptome response to ‘*Ca*. L. solanacearum’ (Lso) infection using RNA-Seq technology. The main goal was to identify early genes that are specifically up-regulated by Lso in these hosts. For this purpose the potato and tomato transcriptome of plants inoculated with Lso-free *B. cockerelli* (Lso-) was compared with that of plants inoculated with Lso-infected *B. cockerelli* (Lso+). In potato, a list of 138, 71 and 86 genes were found to be differentially up-regulated (logFC >1, FDR<0.05) in response to Lso at 24 h, 72 h and 10 days post inoculation, respectively. The majority of these genes (98%) were specific and not shared among time points. Interestingly, more differentially-expressed genes (DEGs) were found to be down-regulated (525) than up-regulated (289) in response to Lso at all time points combined. Since our approach is based on early induction of the plant immune response we focused on genes that were specifically up-regulated by Lso at the two early time points (24 and 72 h). We narrowed down our list of genes by selecting genes up-regulated in Lso+ compared with both Lso- and with untreated control treatments. We found 27 and 8 genes at the 24 and 72 h time points, respectively (Table 1). Unfortunately, our tomato RNA-Seq data did not allow identification of differential gene expression at the 24 h time point, due to large variation and overlap in the Lso+, Lso- and control treatments. Six potato DEGs were tested directly by qPCR and showed the same expression pattern as in the RNA-Seq. These, validated, Lso-specific up-regulated genes were also searched in the databases and some were found to be induced by biotic stress only. Therefore, these genes will serve as good candidates for the induced HR approach.

For our second objective several variations of media and coated-glass surfaces were tested to improve Las attachment to microfluidic flow chamber (MCF) surfaces. The most dramatic improvement of bacterial attachment was obtained by modifying the composition of culture media and using the model bacterium *Liberibacter crescens* (Lcr). Las and Lso were also tested in a newly designed MFC, but although attachment improved, media still needs to be refined to achieve strong cell attachment.

In the frame of this project we generated a transcriptomic profile of both tomato and potato in response to *B. cockerelli* and to Lso-infected *B. cockerelli* and identified putative Lso-specific host induced genes. The promoters of these genes will be used to generate the HR-inducing constructs described in our full proposal. Furthermore, our progress in Las cultivation in MFC will allow us to conduct unique experiments on Las under *in vitro* conditions with plant mimicking conditions.

Agricultural and/or economic impacts of the research findings.

The main goal of this research proposal was to set the stage for examining a new approach to control *Ca*. L. spp. diseases and to establish a new methodological platform to study *Ca*. L. spp. *in vitro*. Hence, to fulfill its potential agricultural and economic impact this research needs to be continued as was describe in the feasibility proposal for the 2nd and 3rd years. In this regard, we can conclude that the goals set for the period of the feasibility study were fully achieved and that the necessary knowledge has been obtained. A continuation proposal, based on the hypothesis proposed in the feasibility proposal will be submitted this year to BARD for the regular grant program.

Contribution of the collaboration: whether and how project objectives were promoted as a result of the cooperation.

The goals of this research proposal were impossible to achieve without the collaboration of the three groups (Volcani Center, Auburn University and UC Davis). The zebra-chip patho-system, including the suspected agent Lso (haplotype A/B), and the vector *B. cockerelli* are only available for the UC Davis team and therefore they setup the main potato and tomato inoculation experiments. The team in Volcani Center included a specialized bioinformatician that was able to analyze the data generated from the RNA-Seq analysis and this data was further studied by a dedicated student in the lab. The team in Auburn University is one of few in the world with expertise in using microfluidic technology to study vector-transmitted plant bacterial pathogens. Therefore, this small consortium was able to use different expertise, technology and to bridge geographical limitations to successfully fulfill the goals of this proposal.

RESEARCH ACHIEVEMENTS

The two main goals of this feasibility research were to:

I. Identify and validate '*Ca*. L.'-specific host induced genes that would serve as candidate for the promoter-fusion induced HR approach.

II. Establish and optimize a microfluidic-based system for *in vitro* cultivation and study of *Ca*. L. asiaticus

Goal I.

To achieve goal I we conducted an RNA-Seq analysis of tomato and potato plants inoculated with Lso-infected *B. cockerelli* (Lso+), Lso-free *B. cockerelli* (Lso-) and untreated plants (control). Plant tissue was sampled at one, three and ten days post inoculation and total RNA was purified. Samples were sent for RNA-Seq and then bioinformatically analyzed to identify differentially-expressed genes (DEGs). To the best of our knowledge this is the first RNA-Seq analysis to decipher potato and tomato response to both *B. cockerelli* and Lso. Variation among biological replicates in the tomato experiment was large and it was difficult to extract DEGs. Therefore, in this report we will focus only on the potato RNA-Seq results.

Principal component analysis shows that samples cluster mostly based on sampling time. However, within each time point, the biological replicates cluster based on treatments indicating that each treatment yielded a different host response (Fig. 1). We compared Lso+ and Lso- treatments with the control treatment to identify DEGs (logFC >1, logFC<-1 FDR<0.05). With both comparisons the maximum number of DEGs (up- and down-regulated combined) was at the 72 h time point (Fig. 2). The Lso+ treatment led to nearly two-times higher number of DEGs compared with the Lso- treatment (2406 and 1300 genes, respectively). To differentiate between *B. cockerelli*- and Lso-induced DEGs, we directly compared the transcriptome of the Lso+ and the Lso- treatments. This analysis yielded a set of 245 and 545 up- and down-regulated genes, respectively (Fig. 3). These genes were considered as being Lso-specific. The most pronounced difference was at the 24 h time point with 474 DEGs, compared with 149 and 217 at the 72h and 10 days time points, respectively. Since the focus of this study was on early plant response to Lso, we focused on the 24 and 72 h time points. To narrow down the list of Lso-specific host induced genes even further, we selected genes that were differentially up-regulated when the Lso+ treatment was compared with the Lso- and with the control treatment. This analysis yielded a list of 35 up-regulated genes (Table 1) that are speculated to be specifically-induced by the bacterial pathogen Lso. These genes would serve as good candidates for the promoter-fusion induced HR approach. To test whether the 35 genes

(39 transcripts) in Table 1 are known to be responsive to different treatments we searched gene expression and literature databases. However, since relatively little information is available on potato gene expression we also searched for their Arabidopsis orthologs. Of the 39 transcripts, 17 did not have Arabidopsis orthologs and therefore only the potato gene ID could be searched (Table 2). Of these 17 transcripts only one (PGSC0003DMT400047125) was found in databases, up-regulated in response to *Phytophthora infestans* infection. Ten of the remaining 22 transcripts, which had an Arabidopsis ortholog, were not found in previous publications and therefore there is limited information on their expression behavior. Three transcripts were found to be up-regulated in response to biotic stresses, including one gene found to be up-regulated in response to Las. Five transcripts were up-regulated by abiotic stresses, and four were up-regulated by both biotic and abiotic stresses (Table 2).

Six DEGs (2 up and 4 down-regulated genes) were further tested by qPCR with specific primers. All six showed the same expression patterns as in the RNA-Seq (Fig. 4), providing further support for the validity of the RNA-Seq results.

Additional analyses to learn on early potato response to *B. cockerelli* and Lso were conducted. These include GO enrichment (Figs. 5 and 6) and pathway analyses (Fig. 7), both showing differential regulation of many stress-related GOs and pathways in response to Lso+ and Lso- treatments. Since these analyses are not the main focus of this report and due to lack of space, it will not be further discussed.

In summary, our goal to identify and validate early, Lso-specific, host induced genes was achieved and we now have a list of ~30 candidate genes for the promoter-fusion induced HR approach.

Goal II.

Ca. L. spp. cultivation remains a challenge and no media has yet been successfully devised for that purpose. In goal II we aimed to examine the feasibility of using the microfluidic flow chamber (MFC) technology, for *Ca*. L. spp. study and cultivation. One of the main limitation in culturing Lcr, Las and Lso in MFC is to achieve attachment of the bacteria to MFC surface. Using the only culturable member of the *Liberibacter* genus so far, Lcr, we tested different chemicals to coat the glass surfaces used in MFC, without success. By switching fetal bovine serum (FBS) with Methyl-β-cyclodextrine in BM7 media (modified BM7, mBM7), we were able to not only increase Lcr cell attachment, but also see development of cell aggregates that resemble biofilm formation (Fig. 8A). This effect was characterized in MFC and is exemplified by an increase in Lcr attachment going from

no cells attached in BM7 to an adhesion force of 117 ± 15 pN using mBM7. When Las and Lso cells were introduced using different media than mBM7 (Fig. 8B and C), as well as different types of MFC, including a newly designed MFC model (Fig 9A), attachment was weak and happened mostly in areas without constant flow (Fig. 9B and C). Attempts to use mBM7 for initial Lso and Las attachment (this media does not allow Las or Lso growth), before switching to another medium in MFC are underway. The most interesting achievement for this goal during this feasibility project was the finding that modifying media components drastically change cell attachment, therefore we will use this approach, as well as our newly designed MFC, to follow up this work.

Publications for Project IS-4841-15

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun

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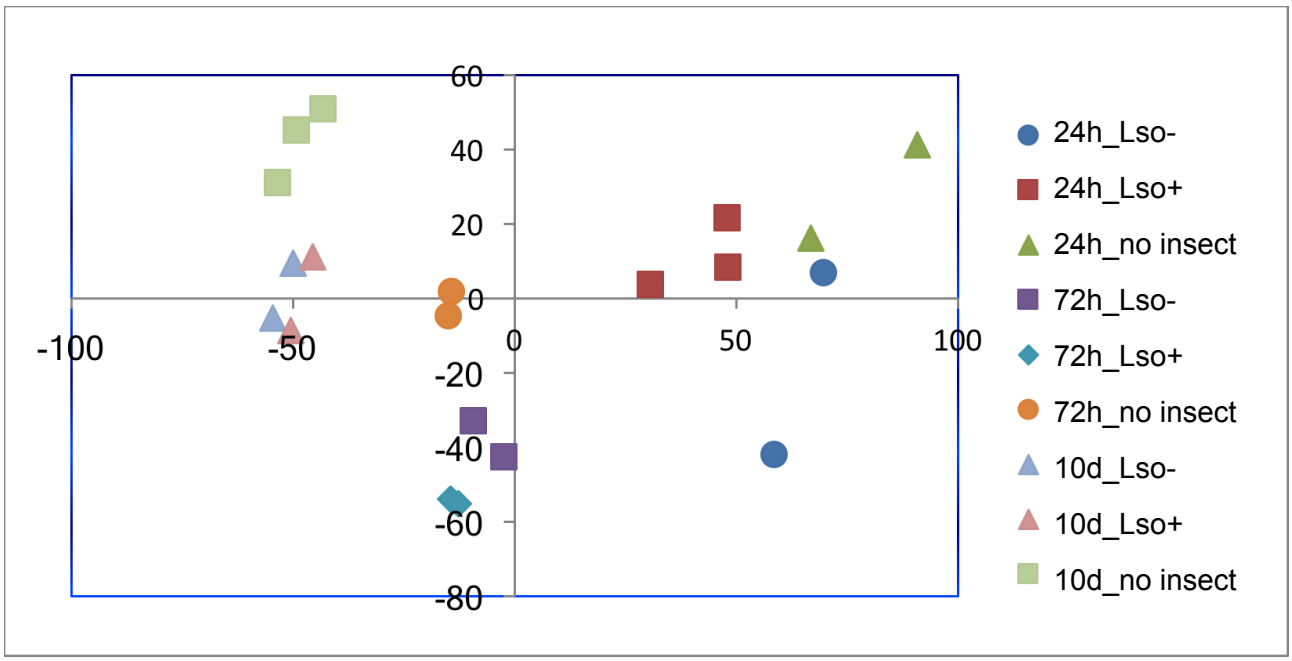
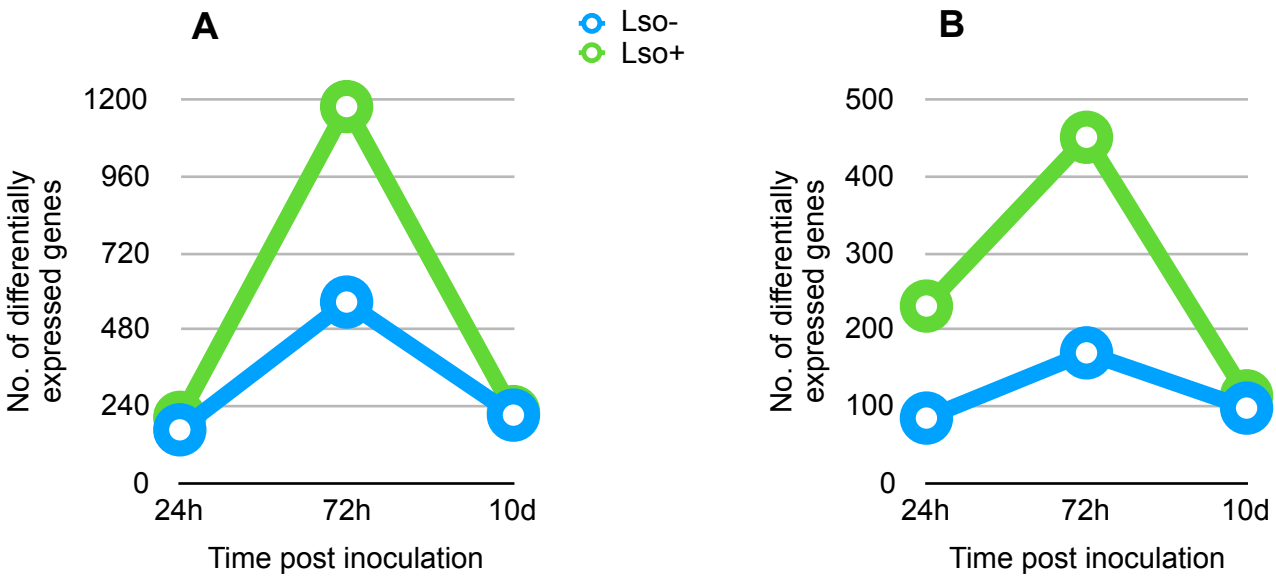
FIGURE 1



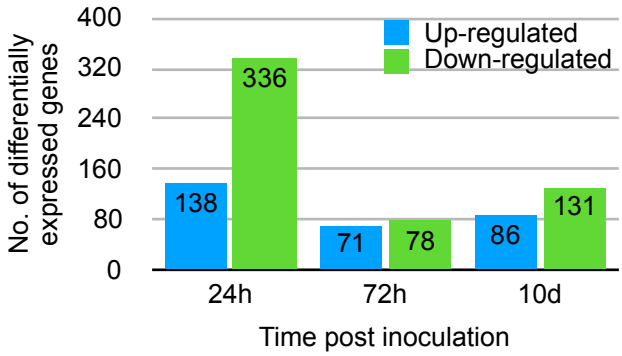
Principal component analysis of potato RNA-seq in response to inoculation with Lso-infected *B.cockerelli* (Lso+), Lso-free *B. cockerelli* (Lso-) and untreated control plants (no insect) at 24 h, 72 h and 10 days after inoculation.

FIGURE 2



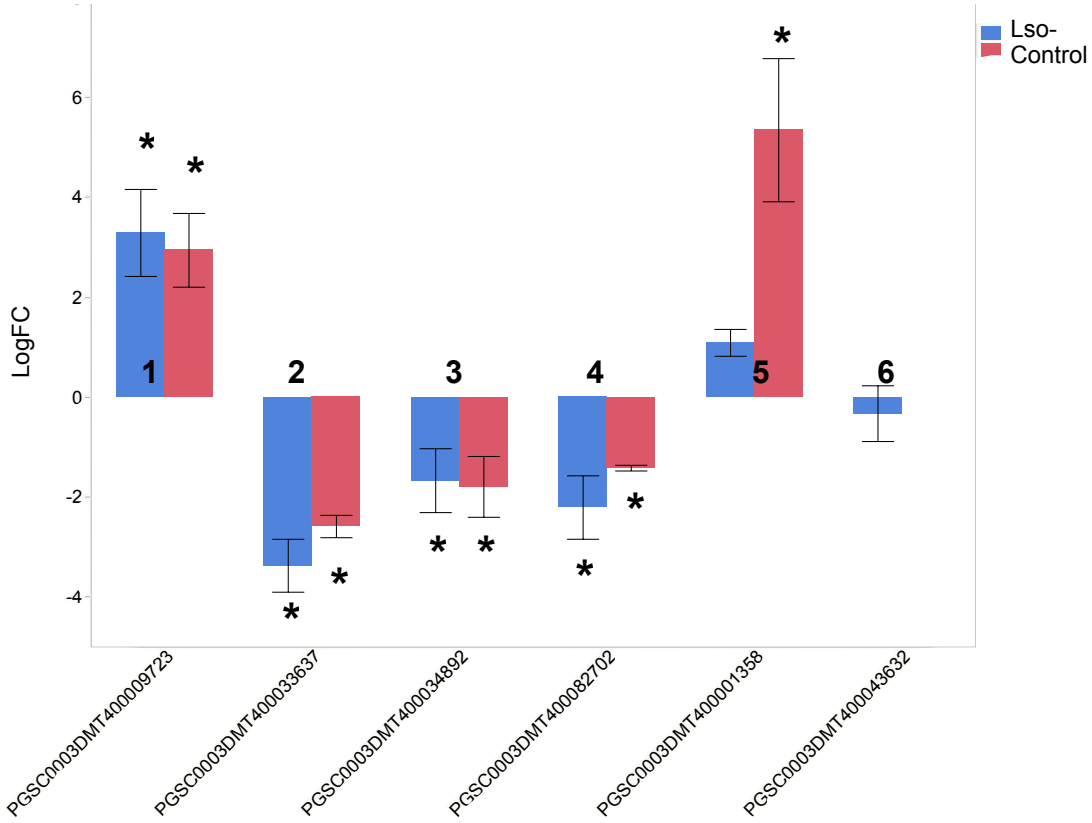
Temporal analysis of potato up- **(A)** and down-regulated **(B)** genes in response to Lso-infected *B.cockerelli* (Lso+) and Lso-free *B. cockerelli* (Lso-). Potato DEGs of Lso+ (green line) and Lso- treatments were compared with untreated control samples. The number of DEGs (logFC >1, logFC<-1 FDR<0.05) over time was extracted.

FIGURE 3



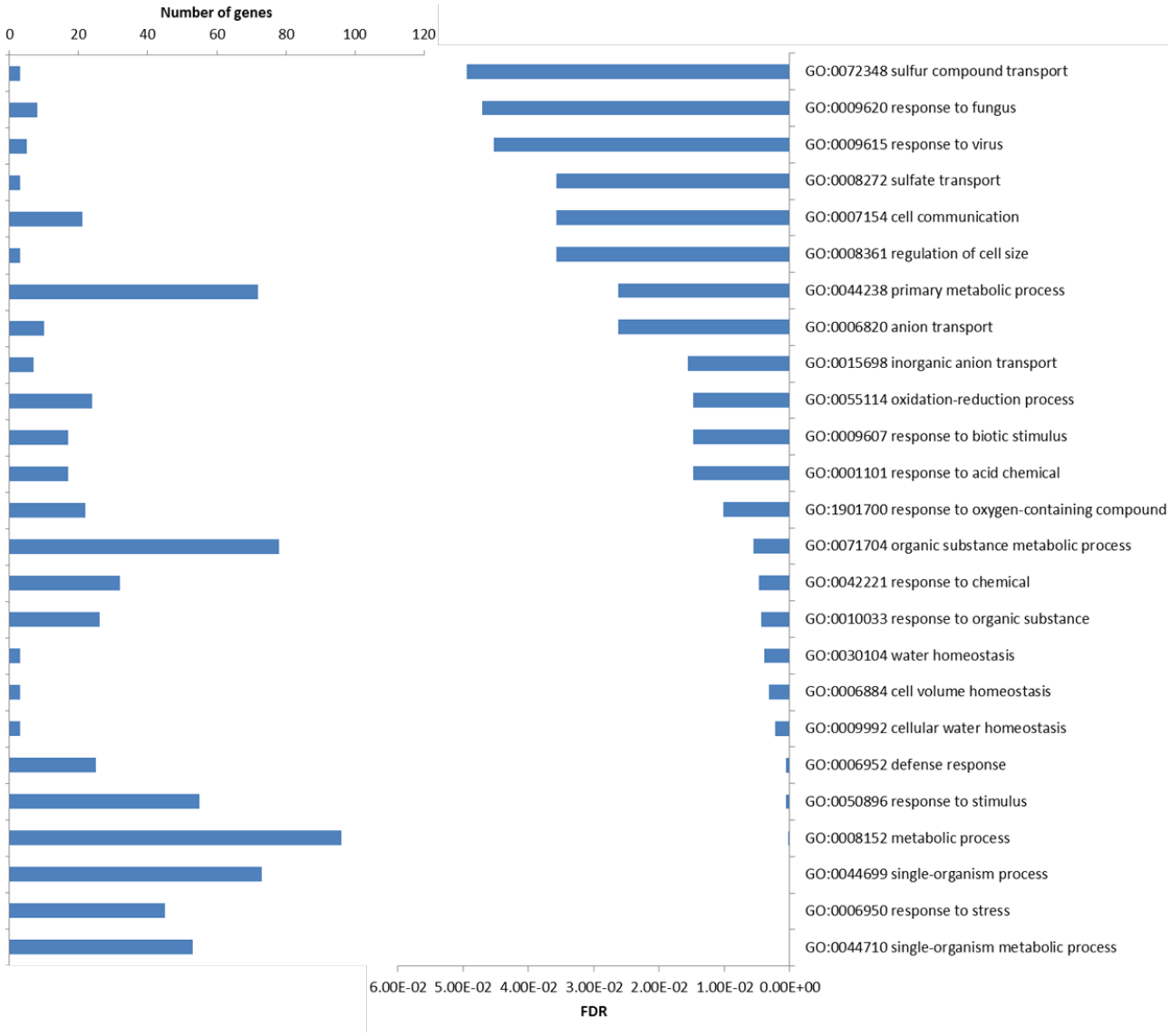
Number of potato up- (blue) and down-regulated (green) genes induced by Lso. Potato transcriptional response to Lso+ and Lso- treatments was compared and DEGs were extracted.

FIGURE 4



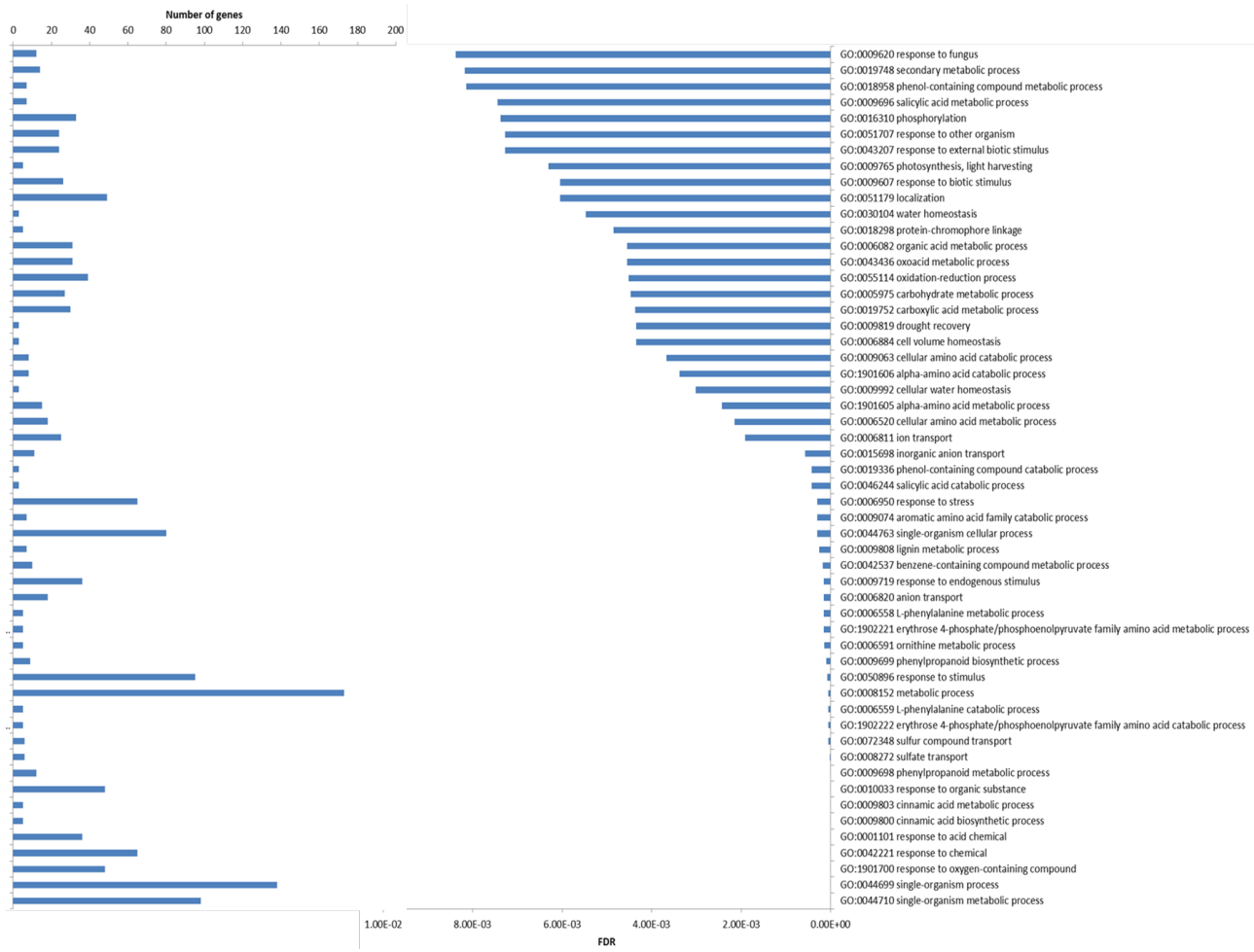
Validation of potato DEGs by quantitative-PCR. Six potato DEGs (1, 5: up-regulated; 2-4, 6: down-regulated) were analyzed by qPCR with specific primers. Actin was used as normalizing gene. Log fold-change (LogFC) was determined using the ΔΔct method. All six gene had the same expression pattern as in RNA-Seq. The two up-regulated genes (1 and 6) are in bold in Table 1. Asterisks indicate significant difference ($p<0.05$) between Lso+ and the indicated treatment (t-test).

FIGURE 5



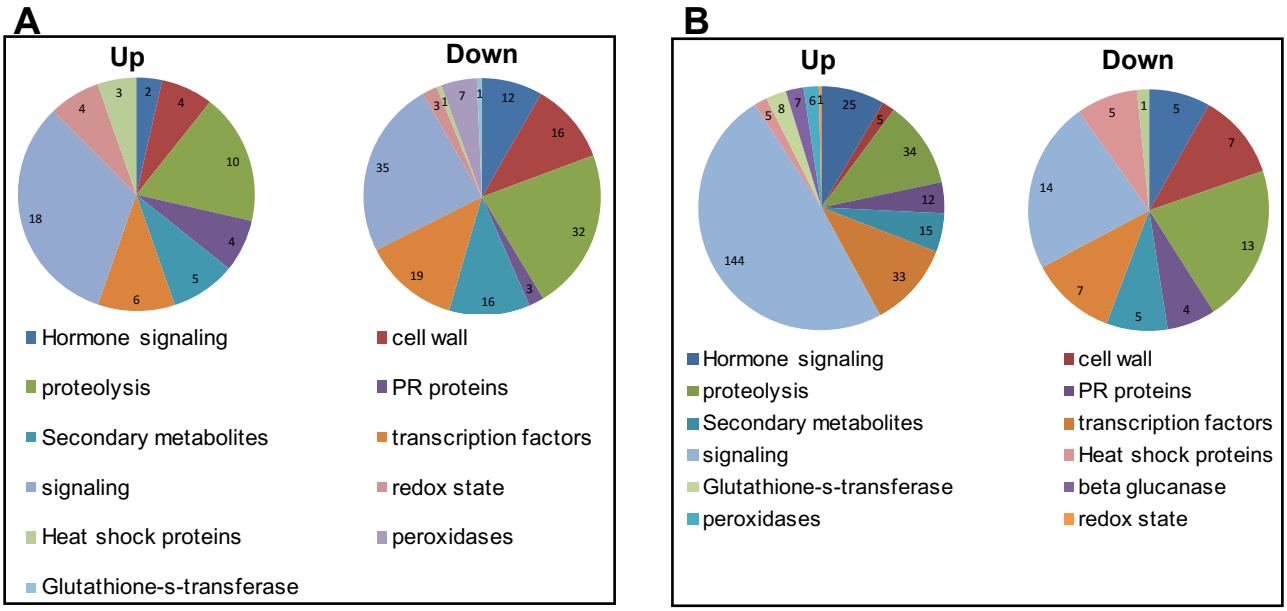
Gene ontology (GO) enrichment (FDR<0.05) of potato genes up-regulated by Lso-treatment 24 h post inoculation. All up-regulated potato genes in response to Lso-treatment were submitted to GO analysis using Blast2GO. GO names and FDR are indicated on the right and the number of genes associated with each GO is on the left.

FIGURE 6



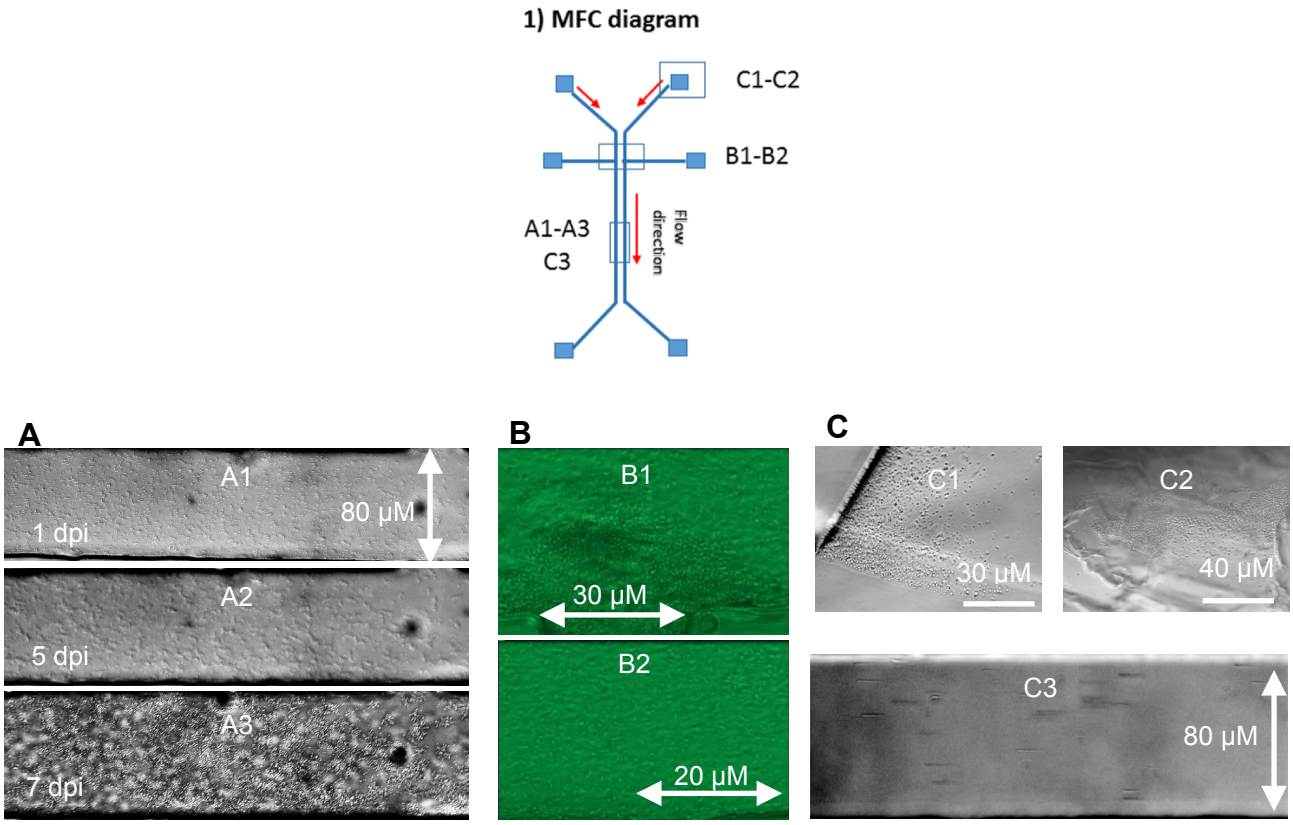
Gene ontology (GO) enrichment (FDR<0.05) of potato genes down-regulated by Lso+ 24 h post inoculation. All down-regulated potato genes in response to Lso+ treatment were submitted to GO analysis using Blast2GO. GO names and FDR are indicated on the right and the number of genes associated with each GO is on the left.

FIGURE 7



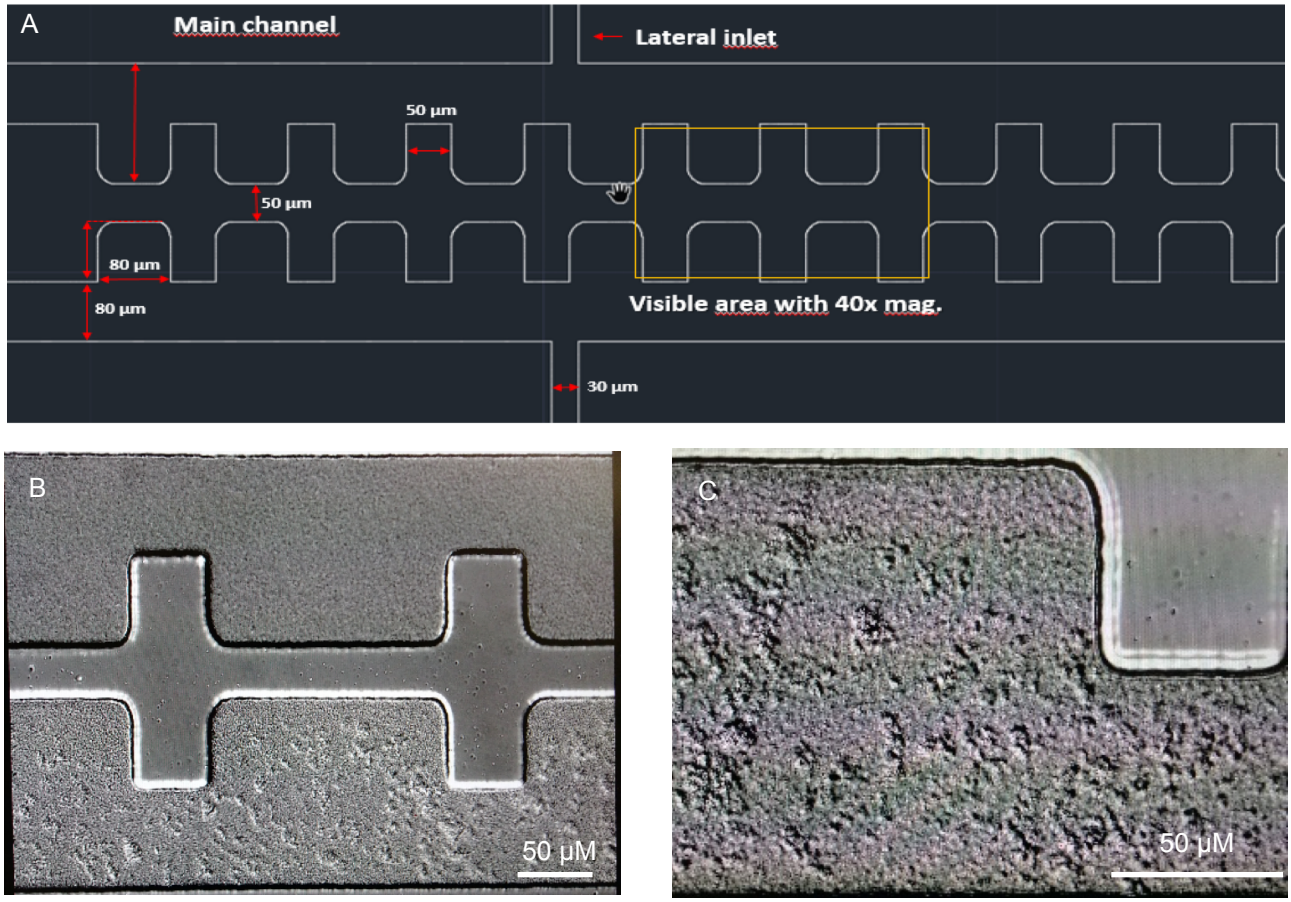
Biotic stress pathways up- and down-regulated in response to Lso during the first 72 h. All up- (left pie) and down-regulated (right pie) potato genes in response to Lso+ **(A)** and Lso- **(B)** were submitted to the MapMan tool to identify the molecular pathways whose expression were altered in response to Lso+ and Lso- treatments. This analysis shows only the breakdown of the biotic stress pathway expression changes in response to Lso+ and Lso- treatments.

FIGURE 8



Liberibacter spp. assays in MFC. 1) Diagram of MFC used for these experiments. Labeled boxes correspond to the areas were images with the same names were taken. **(A)** Lcr attachment and growth in microfluidic chambers over time using mBM7. **(B)** Lso aggregate formation in MFC 24 h after inoculation using modified Mueller-Hinton broth. Infected potato tubers provided by the Clare Casteel's lab (UC Davis partner in this BARD project), and confirmed by qPCR, were used as initial inoculum for a media formulation based in the Mueller-Hinton broth supplemented with ascorbic acid. Non-motile small pleiomorphic coco-bacilli were observed forming a consistent cell aggregate in the lateral inlets, as well as attaching to the main channels. **(C)** Las cells observed in HLB-infected citrus samples (confirmed by qPCR) inoculated in MFC using G50 medium (Parker et al., 2014). Non-motile small pleiomorphic coco-bacillary cells were observed flowing inside MFC.

FIGURE 9



Novel MFC designed to improve bacterial cell capture and attachment. **(A)** Schematic presentation of a new microfluidic chamber design, drawn using AUTOCAD® 2015. **(B)** Zoomed lateral inlet intersection area in the new design. A set of cavities were included in the main channels to retain planktonic cells. Each cavity is 80 x 80 µm separated with a 50 µm section. Main channels and lateral inlets dimensions were maintained to 80 and 30 µm respectively as the main channel separator was set to 50 µm. The design allows to simultaneously asses two cavities of each channel with the inverted microscope, when used at 40x magnification. **(C)** Lcr cell attachment and cell aggregate formation in the new MFC design. The new design showed a higher cell retention when tested with Lcr and it is been tested with Las.

TABLE 1

Lso-infected *B. cockerelli* induced genes compared with Lso-free *B. cockerelli* and untreated control plants at 24 and 72 h post inoculation.

TPI* (h)	Protein ID	Protein description	logFC Vs. control	PValue	logFC Vs. *B. cockerelli*	PValue
24	16741	Gtpase mss1/trme	11.12	0.0000	11.13	0.00002
24	35005	Cytochrome P450	10.86	0.0000	10.86	0.00000
24	31338	Armadillo/beta-catenin repeat family	10.62	0.0000	10.63	0.00004
24	08481	Hsp70-interacting protein 1	10.39	0.0000	10.39	0.00000
24	25297	Dimethylallyltransferase	9.73	0.0000	5.32	0.00000
24	05377	Cellular nucleic acid binding protein	9.56	0.0000	9.56	0.00000
24	16024	Pentatricopeptide repeat-containing	9.52	0.0002	9.52	0.00034
24	19492	DNA-damage-inducible protein f	9.45	0.0003	9.45	0.00046
24	02475	Phospholipase C	9.20	0.0004	9.21	0.00066
24	20043	Protein phosphatase-2c	9.05	0.0000	9.04	0.00001
24	31873	Vetispiradiene synthase	8.73	0.0000	8.72	0.00001
24	47855	Conserved gene of unknown	8.61	0.0000	5.37	0.00039
24	19226	R2r3-myb transcription factor	8.53	0.0001	8.55	0.00019
24	08360	Trichohyalin	8.31	0.0000	4.37	0.00027
24	06678	Ornithine carbamoyltransferase	8.12	0.0000	8.12	0.00009
24	03631	Bipolar kinesin KRP-130	7.96	0.0001	7.96	0.00016
24	06399	Zinc finger protein	7.75	0.0002	7.75	0.00033
24	56332	ATP binding protein	7.74	0.0002	7.74	0.00033
24	24687	Flavonoid 3-hydroxylase	7.66	0.0004	7.66	0.00065
24	56120	Fatty acid desaturase	5.60	0.0000	3.18	0.00056
24	06970	Copper chaperone	5.35	0.0000	2.96	0.00073
24	07852	Cc-nbs-lrr resistance protein	4.18	0.0002	4.82	0.00004
24	54885	Glycine-rich protein A3	4.10	0.0000	5.12	0.00003
24	**06773**	**Gene of unknown function**	**2.97**	**0.0002**	**3.26**	**0.00049**
24	47960	Superoxide dismutase	2.64	0.0000	1.87	0.00003
24	35817	DNA-binding protein 3	2.02	0.0000	1.86	0.00053
24	49121	Soul heme-binding family protein	1.91	0.0004	5.45	0.00000
72	09149	Conserved gene of unknown	11.52	0.0000	11.50	0.00000
72	45905	ATP binding protein	9.49	0.0000	9.47	0.00000
72	02575	Nucleic acid binding protein	9.12	0.0000	9.10	0.00000
72	40575	Cleft lip and palate transmembrane	8.40	0.0000	8.38	0.00009
72	29708	Nitrate transporter	4.24	0.0000	3.39	0.00000
72	**01000**	**Alpha-DOX2**	**3.19**	**0.0000**	**10.31**	**0.00000**
72	49866	Acyl-protein thioesterase	2.56	0.0017	1.76	0.00000
72	13477	Urea active transporter	2.14	0.0000	2.01	0.00001

*time post inoculation (hours)

**All potato protein ID start with PGSC0003DMP4000

TABLE 2

Gene expression data collected from the literature related to the 35 Lso-specific genes listed in Table 1.

Protein ID*	Transcript ID**	Arabidopsis gene ID	% similarity	Response to biotic stress	Response to abiotic stress
06970	09996	AT1G12520	61.86	Up: sucking insects	Up: salt, As
29708	43794	AT1G12940	70.20	N/A	N/A
49866	73687	AT1G52700	73.15	N/A	N/A
06678	09590	AT1G75330	73.26	N/A	Up: Zn, ABA
16741	24486	AT1G78010	61.74	N/A	N/A
19492	28665	AT2G38330	65.24	N/A	N/A
01000	01358	AT3G01420	70.77	Up: Alternaria brassicicola	Up: phosphate
	73687	AT3G15650	73.15	Up: Ca. L. asiaticus	N/A
02575	03613	AT3G16220	44.32	N/A	N/A
20043	29442	AT3G16560	56.62	N/A	Up: nitrate
05377	07741	AT3G43590	37.78	N/A	N/A
19226	28230	AT3G46130	44.19	Up: Pectobacterium, Phytophthora	Up: nitrogen,CO_2
25297	37239	AT4G17190	71.57	Up: Plutella xylostella	N/A
08481	12204	AT4G22670	72.58	Down: P. syringae pv. tomato	Up: salt
40575	60288	AT5G08500	74.33	N/A	N/A
	60288	AT5G23575	70.33	N/A	N/A
16024	23499	AT5G40400	47.69	N/A	N/A
13477	19715	AT5G45380	85.11	N/A	Up: nitrogen
	37239	AT5G47770	71.57	N/A	N/A
47960	70920	AT5G51100	58.33	N/A	Up: Arsenic
	28230	AT5G59780	42.44	N/A	Up: SA, ethylene, JA, salt
31338	46296	AT5G67340	43.97	Up: flg22, Botrytis cinerea	N/A
56332	84310	N/A		N/A	N/A
56120	83859	N/A		N/A	N/A
54885	80961	N/A		N/A	N/A
49121	72620	N/A		N/A	N/A
47855	70789	N/A		N/A	N/A
45905	67973	N/A		N/A	N/A
35817	53122	N/A		N/A	N/A
35005	51968	N/A		N/A	N/A
31873	47125	N/A		Up: Phytophthora infestans	N/A
24687	36400	N/A		N/A	N/A

09149	13198	N/A		N/A	N/A
08360	12009	N/A		N/A	N/A
07852	11298	N/A		N/A	N/A
06773	09723	N/A		N/A	N/A
06399	09232	N/A		N/A	N/A
03631	05131	N/A		N/A	N/A
02475	03474	N/A		N/A	N/A

*All potato protein ID start with PGSC0003DMP4000

**All potato transcript ID start with PGSC0003DMT4000